Eplerenone With Valsartan Effectively Reduces Atherosclerotic Lesion by Attenuation of Oxidative Stress and Inflammation

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Objective—Angiotensin II contributes to atherogenesis, mainly through oxidative stress and inflammation. Recent data suggest that aldosterone is implicated in some effects of angiotensin II. We hypothesized that aldosterone could directly contribute to oxidative stress and atherosclerotic lesion formation.

Methods and Results—Male apolipoprotein E–deficient mice 6 weeks of age were placed on a normal diet or 1.25% high-cholesterol diet. After 6 weeks of the high-cholesterol diet, a marked increase in atherosclerotic lesion formation was observed in the aorta, accompanied by significant elevation of plasma cholesterol level. Production of superoxide anion and expression of NAD(P)H oxidase subunit p47phox, tumor necrosis factor-α, and monocyte chemoattractant protein-1 in the aorta were increased with the high-cholesterol diet. Eplerenone (1.67 g/kg in high-cholesterol diet) did not affect blood pressure or plasma cholesterol but decreased the atherosclerotic area by nearly 70% (P<0.05), associated with attenuation of oxidative stress and inflammatory response. Valsartan (0.5 mg/kg per day) also decreased the atherosclerotic lesion, whereas coadministration of valsartan and eplerenone further decreased it. Moreover, aldosterone (0.1 μmol/L) enhanced NADPH oxidase activity in cultured vascular smooth muscle cells.

Conclusions—These results suggest that aldosterone may play a critical role in atherogenesis subsequent to oxidative stress in part independent of angiotensin II–mediated signaling, and that eplerenone could prevent atherosclerosis by attenuating oxidative stress and inflammation. (Arterioscler Thromb Vasc Biol. 2006;26:000-000.)

Key Words: angiotensin II aldosterone receptors oxidative stress atherosclerosis

Oxidative stress attributable to excessive production of reactive oxygen species (ROS) such as superoxide plays a central role in the development and progression of atherosclerosis because it mediates a wide range of pathological processes including lipid peroxidation, reduction of NO bioactivity, and induction of inflammatory genes. Accumulating evidence indicates that the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex is an important source of ROS in several cardiovascular disease settings. NADPH oxidase complex consists of the membrane-associated flavocytochrome b588 protein, which is composed of nox1, nox4, and p22phox, cytosolic components p47phox and p67phox, and a low molecular weight G-protein, rac-1.

Angiotensin II (Ang II) is the principal vasoactive substance of the renin-angiotensin system, which has a variety of physiological actions including vasoconstriction, aldosterone release, and cell growth. In various vascular cell types, Ang II is a potent mediator of oxidative stress through activation of NADPH oxidase, which contributes to the development of atherosclerosis. Recently, several lines of data have suggested that aldosterone is implicated in some of the effects of Ang II. Aldosterone potentiates ROS-dependent extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase signaling induced by Ang II in vascular smooth muscle cells (VSMCs). Animal studies using hypercholesterolemic rabbits, Ang II–treated rats, and aldosterone-treated mice showed that an aldosterone receptor antagonist reduced oxidative stress and attenuated vascular changes. These observations suggest that aldosterone is involved in the pathogenesis of atherosclerosis in part independent of Ang II–mediated signaling. However, the direct effect of aldosterone on oxidative stress and how aldosterone contributes to spontaneous atherosclerosis are still unclear.

In the present study, we hypothesized that aldosterone could directly induce oxidative stress and contribute to the pathogenesis of spontaneous atherosclerosis. To test this hypothesis, we examined the effect of eplerenone, a selective aldosterone receptor antagonist, on oxidative stress, inflammation, and atherosclerotic lesion formation in apolipoprotein E (apoE)–defi-
cient mice. We also examined the effect of combination therapy of eplerenone and an Ang II type 1 (AT₁) receptor blocker, valsartan, on lesion formation. In addition, the effect of aldosterone on NADPH oxidase activity in cultured rat VSMCs was also examined.

Materials and Methods

Animals and Treatment
Adult male apoe-deficient mice (The Jackson Laboratory; Bar Harbor, Me) 6-week-old were used in this study. The animals were housed in a room where lighting was controlled (12 hours on, 12 hours off) and the temperature was kept at 25°C. They were given a standard diet (donated by Pfizer Inc., New York, NY) or high-cholesterol diet (HCD; 1.25% cholesterol and 10% coconut oil in standard diet, donated by Pfizer Inc.) for 10 weeks from 6 weeks of age and water ad libitum. Eplerenone (1.67 mg/kg in HCD; donated by Pfizer Inc.) was administered orally from 6 weeks of age. Valsartan (donated by Novartis Pharma AG; 0.5 mg/kg per day) was administered using an osmotic minipump (Alzet model 1002; Durect Corp.) implanted intraperitoneally from 6 weeks of age as described previously.11 Systolic blood pressure was assessed by noninvasive tail-cuff system (MK-1030; Muromachi Kikai Co., Ltd.) in conscious mice. All animal studies were reviewed and approved by the animal studies committee of Ehime University.

Plasma Cholesterol and Aldosterone Levels
Plasma cholesterol level was measured by cholesterol oxidase method (Cholesterol E-test; WAKO Chemical Industries, Ltd.). Plasma aldosterone level was measured using an ELISA kit (Alpha Diagnostic International Inc.).

Atherosclerotic Lesion Area
Mice were euthanized at 12 weeks of age, and the atherosclerotic lesions were analyzed as described previously.12 The proximal portion of the thoracic aorta up to the aortic origin (aortic arch) was taken, and freshly frozen sections were prepared and stained by oil-red O. The aorta from aortic arch to iliac bifurcation was excised and opened longitudinally. Color images were taken with a digital camera (PIX4500; Nikon) connected to a computer. Atherosclerotic lesions area was quantified by analyzing the open luminal surface of oil red O-stained aorta with Densitograph imaging software (Densitograph; ATTO Corp.). The amount of lesion formation in the entire aorta in each animal was measured as the percentage of lesion area per total area of the aortic endothelial surface.

Superoxide Detection
Histological detection of superoxide was performed as described previously.13 In brief, frozen, enzymatically intact, 10-μm-thick sections of cross-sections of the proximal aorta were incubated with dihydroethidium (DHE; 10 μmol/L) in PBS for 30 minutes at 37°C in a humidified chamber protected from light. DHE is oxidized on reaction with superoxide to ethidium bromide, which binds to DNA in the nucleus and fluoresces red. For endothium bromide detection, a Zeiss Axioskop microscope (Axioskop 2 plus with AxioCam; Carl Zeiss) equipped with a computer-based imaging system was used. The intensity of the fluorescence was measured and quantified by use of computer imaging software (Densitograph; ATTO Corp.).

Immunohistochemical and Immunofluorescent Study
Immunohistochemical staining was performed on freshly frozen sections by streptavidin-biotin-peroxidase technique. Anti–tumor necrosis factor-α (TNF-α) and anti–monocyte chemoattractant protein-1 (MCP-1) antibodies were purchased from Santa Cruz Biotechnology Inc. Macrophage was stained with anti-F4/80 antibody (BMA Biomedicals AG). Immunofluorescence was also assessed using freshly frozen sections. Sections were incubated with anti-p47phox antibody (Santa Cruz Biotechnology), washed, and incubated with biotin-labeled secondary antibodies, then incubated with Cy3-labeled streptavidin. Serial sections treated with secondary antibodies alone did not show specific staining. Samples were examined with a Zeiss Axioskop microscope equipped with a computer-based imaging system. The intensity of the fluorescence was analyzed and quantified by use of computer imaging software (Densitograph; ATTO Corp.).

Real-Time RT-PCR
Total RNA was extracted from the aorta. After reverse transcription reaction, real-time quantitative RT-PCR was performed with a SYBR green kit (SYBR Premix Ex Taq; Takara Bio Inc.) as described previously. PCR primers for MCP-1 were 5'-GTCCCTGCAATCTATCTGGGA-3' (forward) and 5'-GAGACATTCGTCCTCCTCA-3' (reverse). PCR primers for p47phox were 5'-GTCCCTGCAATCTATCTGGGA-3' (forward) and 5'-GGAGACATTCGTCCTCCTCA-3' (reverse).

Cell Culture and Treatment
Adult rat aortic VSMCs were prepared and cultured as described previously.14 Subconfluent VSMCs from passage 5 to 7 were used in the following experiments. Before the experiments, VSMCs were kept for 24 hours in quiescent medium without serum. Then the cells were treated with DMEM containing aldosterone (0.1 μmol/L; Sigma Aldrich Corp.) with or without actinomycin D (5 μg/mL; Sigma Aldrich Corp.) as indicated in each experimental condition.

NADPH Oxidase Activity
NADPH oxidase activity was measured as described previously with slight modifications using 5 μmol/L lucigenin (Sigma Aldrich Corp.).15 Chemiluminescence was monitored by a luminometer (AB-2200, ATTO Corp.) for 5 minutes after the addition of cell extract.

Statistical Analysis
Values are expressed as mean±SE in the text and figures. Data were analyzed by 2-way ANOVA. If a statistically significant effect was found, post hoc analysis was performed to detect the difference between the groups. A value of P<0.05 was considered statistically significant.

Results

Systolic Blood Pressure and Plasma Cholesterol and Aldosterone Levels
We first examined the effects of a 1.25% HCD or eplerenone (200 mg/kg per day) treatment for 6 weeks on systolic blood pressure and plasma cholesterol and aldosterone levels. As shown in the Table, both plasma cholesterol and aldosterone levels were significantly higher in apoE-deficient mice after 6 weeks of feeding a HCD than in those with a normal standard diet (ND). Eplerenone treatment at this dose did not influence either systolic blood pressure or plasma cholesterol level,
lesions, superoxide production and expression of p47phox, stress and inflammation in the exaggeration of atherosclerotic as atherosclerosis. To assess the involvement of oxidative tant contributors to the pathogenesis of vascular diseases such as atherosclerosis. Oxidative stress and inflammation are thought to be impor-

Effect of Eplerenone on Oxidative Stress and Inflammation

Figure 1. Effect of eplerenone and valsartan on atherosclerotic changes in apoE-deficient mice treated with HCD. Animals received an ND or HCD containing 1.25% cholesterol for 10 weeks, and aortic samples were obtained as described in Methods. Eplerenone and valsartan were administered as described in Methods. Top, Representative staining of freshly frozen cross-sections from the proximal aorta using oil red-O with or without treatment with an aldosterone-receptor antagonist, eplerenone, and an angiotensin receptor blocker, valsartan. Bar=100 μm. Magnification ×200. Bottom, Morphological measurement of atherosclerotic lesion area in the entire aorta. Lesion area was measured as described in Methods, and values are expressed as the percentage of lesion area per total area of the aortic endothelial surface. The mean value of 3 intermittent sections was taken as the value for each animal. Values are mean±SE for morphometric measurements (n=6 to 7). Epl indicates eplerenone (1.67 g/kg in HCD); Val, valsartan (0.5 mg/kg per day). *P<0.05 vs ND; †P<0.05 vs HCD without Epl or Val.

whereas it significantly increased plasma aldosterone level (Table). Valsartan and combination of eplerenone and valsartan also did not affect systolic blood pressure (100.4±3.2 mm Hg for valsartan and 98.6±2.8 mm Hg for eplerenone with valsartan).

Effect of Eplerenone or Valsartan on Atherosclerotic Lesion Area

We next analyzed the atherosclerotic lesion area in the aorta. After 6 weeks of feeding an HCD, apoE-deficient mice displayed a significant increase in atherosclerotic lesion area in the aorta (8.2-fold relative to ND; P<0.05; Figure 1). Lesion area was significantly reduced by treatment with eplerenone (32.2% of HCD; P<0.05; Figure 1). Epl indicates eplerenone (1.67 g/kg in HCD); Val, valsartan (0.5 mg/kg per day; 44.5% of HCD; P<0.05). To examine whether eplerenone could further reduce lesion area in apoE-deficient mice treated with valsartan, apoE-deficient mice were treated with eplerenone and valsartan. Lesion area was significantly lower in apoE-deficient mice treated with eplerenone and valsartan than in those treated with valsartan only (P<0.05).

Effect of Eplerenone on Oxidative Stress and Inflammation

Oxidative stress and inflammation are thought to be impor-
tant contributors to the pathogenesis of vascular diseases such as atherosclerosis. To assess the involvement of oxidative stress and inflammation in the exaggeration of atherosclerotic lesions, superoxide production and expression of p47phox, TNF-α, and MCP-1 were evaluated. Superoxide production and expression of NAD(P)H oxidase subunit p47phox were increased in apoE-deficient mice after 6 weeks of feeding an HCD (Figures 2 and 3; supplemental Figure I). Expression of TNF-α, MCP-1, and macrophage infiltration was also increased in apoE-deficient mice after 6 weeks of feeding an HCD (Figure 3; supplemental Figure II). Treatment with eplerenone significantly attenuated these changes (Figure 3; supplemental Figure II).

Figure 2. Effect of eplerenone and valsartan on superoxide production and expression of p47phox in atherosclerotic lesions in apoE-deficient mice treated with HCD. Frozen sections of the proximal aorta were prepared as shown in Figure 1. Superoxide production in the proximal aorta was detected as red fluorescence after incubation with DHE as described in Methods. Top, Representative fluorescent staining of superoxide with DHE in the proximal aorta. Bottom, Morphometry of the fluorescence. Quantitative analysis was performed using imaging software as described in Methods. The mean value of 3 intermittent sections was taken as the value for each animal. Bar=100 mm. Values are mean±SE for morphometric measurements (n=6 to 7). Epl indicates eplerenone (1.67 g/kg in HCD); Val, valsartan (0.5 mg/kg per day). Magnification ×200. *P<0.05 vs ND; †P<0.05 vs HCD without Epl or Val.

Figure 3. Effect of eplerenone on expression of p47phox and MCP-1 in atherosclerotic lesions in apoE-deficient mice treated with HCD. Samples were prepared from the proximal aorta, and quantitative RT-PCR was performed as described in Methods. Values are mean±SE (n=4 to 5). Epl indicates eplerenone (1.67 g/kg in HCD).
Effect of Aldosterone on NADPH Oxidase Activity in Cultured VSMCs

We finally examined the direct effect of aldosterone on NADPH oxidase activity in cultured VSMCs. As shown in Figure 4, aldosterone (0.1 μmol/L) enhanced NADPH oxidase activity with peaks 3 minutes and 1 hour after aldosterone stimulation in VSMCs. To determine whether the aldosterone-induced NADPH oxidase activation is a nongenomic or genomic effect, we used a transcription inhibitor, actinomycin D. Pretreatment with actinomycin D (5 μg/mL) for 60 minutes did not affect the rapid aldosterone-induced effect on NADPH oxidase activity but abolished the later peak of aldosterone-induced NADPH oxidase activation (Figure 4).

Discussion

It has been reported that lack of apoE causes a marked increase in plasma cholesterol-rich remnants and subsequent atherosclerotic lesion formation. Therefore, apoE-deficient mice provide a unique model to analyze the pathogenesis of spontaneous atherosclerosis. In the present study, we demonstrated that eplerenone attenuated the rapid aldosterone-induced oxidative stress and inflammation and reduced subsequent atherosclerotic lesion formation in apoE-deficient mice receiving HCD. In addition, we demonstrated that eplerenone further reduced atherosclerotic lesion area in apoE-deficient mice treated with valsartan. These results suggest that aldosterone in itself exerts proatherogenic effects. In fact, we observed that aldosterone enhanced NADPH oxidase activity in cultured VSMCs. An important feature that emerged in the present study is that aldosterone directly contributed to oxidative stress and atherogenesis in part independent of Ang II–mediated signaling.

It is widely accepted that Ang II, through the AT1 receptor, plays a central role in the pathophysiology of atherosclerosis. Inhibition of HCD-induced atherosclerosis by genetic disruption of the AT1 receptor was confirmed in apoE-deficient mice. Because AT1 receptor stimulation induces synthesis and release of aldosterone, it may be possible that aldosterone is implicated in some of the effects of Ang II. This possibility is supported by a recent in vitro study demonstrating that aldosterone potentiates Ang II–induced ERK and c-Jun N-terminal kinase phosphorylation. Moreover, an animal study has demonstrated that an aldosterone receptor antagonist, spironolactone, improved vascular remodeling in Ang II–infused rats. Accumulating evidence indicates that endothelial cells and VSMCs express corticoid receptors and produce aldosterone. Aldosterone synthesized in the vasculature may contribute to atherogenesis together with Ang II. It has been demonstrated that aldosterone administration to apoE-deficient mice increased atherosclerosis development. Consistent with these findings, we observed that apoE-deficient mice fed an HCD displayed exaggerated atherosclerotic lesions associated with a significant increase in plasma aldosterone level. Although the mechanism by which hypercholesterolemia increases plasma aldosterone level was not investigated in the present study, a possible explanation is that low-density lipoprotein upregulates AT1 receptor expression and exacerbates the effects of Ang II and the release of aldosterone. It is possible that the combined inhibition of atherosclerosis with eplerenone and valsartan is caused by the inhibition of local aldosterone synthesis with valsartan. However, this possibility seems to be low in our study because: (1) the inhibitory actions by combination of both drugs were stronger than those of each drug alone, and (2) plasma level of aldosterone markedly increased by eplerenone even if the local aldosterone production may be inhibited by valsartan. In the present study, the plasma aldosterone level was significantly increased by eplerenone (Table). Although we used nonhypotensive dose of eplerenone, this increase in plasma aldosterone level is probably caused by the significant blockade of aldosterone receptor by this dose of eplerenone.

It has become clear that oxidative stress represents a common pathogenic mechanism for atherosclerosis. A particularly important mechanism for ROS-mediated atherosclerosis appears to be through stimulation of proinflammatory events. Previous reports showed the critical role of inflammation in the atherosclerotic lesion formation. In our study, inflammatory responses in proximal aorta of apoE-deficient mice, such as the production of TNF-α and MCP-1, and macrophage infiltration were inhibited by eplerenone (Figure 3; supplemental Figure II). Therefore, evaluation of oxidative stress in this model may provide further understanding of the inhibitory effect of eplerenone on atherosclerosis. It has been reported that p47phox, a cytoplasmic component of NAD(P)H oxidase, is required for atherosclerotic lesion progression in apoE-deficient mice. These results suggest that ROS production through NAD(P)H oxidase activation...
plays an important role in the development and progression of atherosclerosis. In the present study, we demonstrated that eplerenone attenuated HCD-induced superoxide production as well as expression of p47phox, TNF-α, and MCP-1 (Figures 2 and 3; supplemental Figures I and II). However, the effects of aldosterone on oxidative stress are not yet fully understood. It has been shown recently that aldosterone increased macrophage superoxide release and macrophage-mediated low-density lipoprotein oxidation, suggesting that aldosterone directly induces oxidative stress. Data from the present study support this possibility because we observed that aldosterone directly enhanced NADPH activity with peaks 3 minutes and 1 hour after stimulation in VSMCs. We also demonstrated that pretreatment with actinomycin D abolished only the later peak of aldosterone-induced NADPH oxidation, indicating that aldosterone contributes to oxidative stress through both nongenomic and genomic pathways. Rapid nongenomic pathway of aldosterone seems to be involved in the activation of signaling molecules, such as protein kinase C and transcription factors, and modulates vascular function. We also reported that aldosterone inhibited ERK phosphorylation in cultured VSMCs within a few minutes. Moreover, the possible inter-relationship of nongenomic with genomic influences is also suggested. Therefore, there is a possibility that the nongenomic pathway in vascular remodeling is involved in early changes of atherosclerosis, such as inflammatory response and oxidative stress. Although we did not assess the possibility that the rapid nongenomic effect of aldosterone contributed to our in vivo observations, our results suggest that eplerenone, an inhibitor of the genomic effect, could prevent atherosclerosis by attenuating aldosterone-induced oxidative stress. Moreover, eplerenone enhanced the inhibitory action of valsartan on oxidative stress in atherosclerotic lesion.

In conclusion, the studies reported here indicate that aldosterone contributes to atherogenic lesion formation consequent to oxidative stress and inflammation in part independent of Ang II–mediated signaling, and that eplerenone, a selective aldosterone receptor antagonist, prevents these changes. Our findings provide important insights into the pathogenesis of atherosclerosis and a novel role for eplerenone in the treatment of atherosclerosis.

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**Figure I.** Effect of eplerenone and valsartan on expression of p47phox in atherosclerotic lesions in apoE-deficient mice treated with HCD. Frozen sections of the proximal aorta were prepared as shown in Figure 1. (Upper) Representative immunofluorescent staining of p47phox using anti-p47phox antibody in the proximal aorta. (Lower) Morphometry of the fluorescence. Quantitative analysis was performed using imaging software as described in “Methods”. The mean value of three intermittent sections was taken as the value for each animal. Scale bar shows 100 µm. Values are mean ± SE for morphometric measurements (n=6-7). ND: normal standard diet. HCD: high-cholesterol diet. Epl: eplerenone (1.67 g/kg in HCD). Val: valsartan (0.5 mg/kg/day). Magnification x200. *p <0.05 vs. ND, †p <0.05 vs. HCD without Epl or Val.

**Figure II.** Effect of eplerenone on expression of TNF-α and MCP-1 and macrophage infiltration in atherosclerotic lesions in apoE-deficient mice treated with HCD. Frozen sections of the proximal aorta were prepared as shown in Figure 1, and immunostaining of TNF-α, MCP-1 and macrophage (F4/80) was performed as described in “Methods”. Representative results from 6 to 7 mice are shown. Epl: eplerenone (1.67 g/kg in HCD). Scale bar shows 100 µm. Magnification x200.
Figure II